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EFFECT OF NEUTRAL RED STAIN ON SETTLEMENT ABILITY OF OYSTER PEDIVELIGERS, *CRASSOSTREA VIRGINICA*[1]

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ABSTRACT The effect of neutral red stain on the settlement of oyster *Crassostrea virginica* (Gmelin) pediveligers was examined. Larvae were offered two types of substrate: oyster shell and acetate sheets. Settlement was measured as the proportion of pediveligers settled after 24 hours and analyzed with two-factor ANOVA. Staining did not significantly affect settlement, although settlement onto acetate was much lower than onto oyster shell.

KEY WORDS: vital stain, neutral red, larvae, settlement, competency, *Crassostrea virginica*

INTRODUCTION

Studies of the early life history of bivalve mollusks often require examination and counting of larvae and newly-settled spat. Under some circumstances, this examination can be greatly facilitated by the use of a vital stain with the nearly transparent larvae or spat. It has been shown that a low concentration of neutral red stain colored oyster (*Crassostrea virginica*) larvae a bright red, with no apparent mortality or side effects (Loosanoff and Davis 1947, Manzi and Donnelly 1971).

In some cases, however, the swimming behavior of *C. virginica* pediveliger larvae in the laboratory was perceptibly altered for several hours after being stained with neutral red (unpublished data). Swimming rate decreased, compared to that of larvae without the stain, and there was a tendency for the stained larvae to clump. Because oyster settlement has a large behavioral component (Cranfield 1973), there is a concern that the use of neutral red to stain pediveliger larvae might affect their ability to settle. This article examines the effect of neutral red stain on the settlement of *C. virginica* eyed pediveliger larvae onto two types of substrates.

MATERIALS AND METHODS

Crassostrea virginica pediveliger larvae were cultured in filtered York River water at 15 ppt salinity.[2] Visual examination of the larvae confirmed that more than 95% had both a foot and a large, distinct eyespot, which are considered morphological characteristics of larval competency to settle (Coon et al. 1985).

Manzi and Donnelly (1971) recommend culture concentrations of neutral red stain of 2.5–5.0 ppm for 24 hours in both distilled water and filtered seawater. In seawater however, neutral red stain forms a precipitate within several days, while solutions in distilled water can be kept at room temperature for months while no perceptible precipitation. Distilled water solutions were used in this experiment.

The larvae were held in 15 ml culture dishes (watch glasses), filled with 10 ml of 0.2 micron-filtered York River water at 15 ppt salinity. Salinities used by Loosanoff and Davis (1947) or Manzi and Donnelly (1971) were not given. Two settlement substrates were used: small, nearly flat *Crassostrea virginica* valves, and circles of Mylar frosted acetate, both about 2.5 cm in diameter. The shells were scrubbed clean, the ligament was removed, and the concave surface was placed downward in the culture dish. The acetate circles were creased so that the frosted surface was concave, and the concave surface placed downwards in the culture dish. Mylar acetate has been used previously as a commercial settlement substrate (Dupuy et al. 1977).

Three days prior to the experiments, the shells and acetate circles were placed in a flowing seawater trough with adult *C. virginica*. Dissolved substances from both oyster shells (Vietch and Hidu 1971) and the common marine bacterium *Alteromonas* (Fitt et al. 1990) enhance settlement of oyster larvae. Exposure to seawater provided substrate accumulation of chemical substances which induce larval settlement. 3–4 Days of exposure permits optimal bacterial growth for settlement inducement (Fitt et al. 1990).

The experimental design involved a two-factor analysis of variance (Zar 1984). Factors were substrate treatment (shell versus acetate) and stain treatment (stain versus no stain), and each treatment combination had five replicates. Approximately 100 pediveliger larvae were added to each culture dish with a dropper pipette. Then three drops of the 0.1 ppt neutral red stain solution were added to the stain-treatment dishes, yielding a culture stain concentration of approximately 3 ppm. All of the culture dishes were covered with glass petri dishes, and covered with a dark cloth for 24 hours. The temperature was constant at 20°C.

At the end of 24 hours, the number of settled spat and free-swimming larvae were counted in each settlement chamber. There were no more than one or two dead larvae per culture dish; most of these had been accidentally crushed by handling. One culture dish was lost; the missing value was estimated using the Shearer technique (Zar 1984). Settlement was expressed as a proportion in each chamber. Prior to statistical analysis, each value was transformed by the arcsine square root method, to bring the data distribution closer to a normal distribution and satisfy the assumptions of analysis of variance (Zar 1984).

RESULTS

Substrate type strongly affected settlement (Tables 1, 2). Mean proportional settlement on oyster shell was 64.2%, compared to only 1.4% on acetate. Staining did not significantly affect settlement (Tables 1, 2); the difference in mean proportional settlement between treatments was only 2.3%. The interaction effect was not significant (Table 2).

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[2]Virginia Institute of Marine Science oyster hatchery.

TABLE 1.

Settlement of oyster larvae, expressed as proportions of total larvae.

Oyster Shell		Mylar Acetate	
Stain	**No Stain**	**Stain**	**No Stain**
0.66	0.63	0.03	0.00
0.73	0.66	0.00	0.06
0.71	0.72	0.00	0.01*
0.36	0.82	0.00	0.03
0.82	0.31	0.01	0.00

* Denotes estimated value: see text.

TABLE 2.

Two factor analysis of variance.

Source	DF	SS	MS	F	p
Stain	1	0.0005	0.0005	0.022	0.887
Substrate	1	3.6607	3.6607	157.8	<0.0001
Interaction	1	0.0079	0.0079	0.341	0.586
Error	16	0.3713	0.0232		
Total	19	4.0403			

DISCUSSION

The hypothesis that staining with neutral red affects settlement of *C. virginica* larvae was not substantiated, with an extremely small (2.3%) difference between staining treatments. The difference between proportional settlement on the substrate treatments (oyster shell versus acetate) shows the degree to which settlement of oyster larvae can be affected.

Although settlement was not affected by neutral red stain, there may be effects on metamorphosis (a physiological event separate from settlement) or spat survival or growth. Metamorphic success and post metamorphic survival is affected strongly by physiological stress (Baker and Mann, in press).

Manzi and Donnelly (1971) examined the effects of neutral red and other stains on larvae of *C. virginica* and the venerid clam *Mercenaria mercenaria*, and reported no difference in survival and growth rates between the two species. *Mercenaria* or other bivalve pediveligers were not used in this study, but based upon Manzi and Donnelly's work, it is reasonable to infer that neutral red will have no significant effect on proportional settlement on other bivalve mollusk taxa.

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